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Ethanol self-administration and reinstatement of ethanol-seeking behavior in *Per1*^{Brdm1} mutant mice

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Abstract
Rationale Alcohol consumption shows circadian rhythmicity, i.e., alcohol preference and intake change with circadian time. Circadian rhythmicity is controlled by a biological clock, which has been shown to govern behavioral, physiological, and hormonal processes in synchronization with internal as well as external cues. Molecular components of the clock include circadian clock genes such as *period* (*Per*) *1*, *2*, and *3*. Previously, our lab demonstrated the involvement of mouse *Per1* (*mPer1*) and *Per2* (*mPer2*) in modulating cocaine sensitization and reward. What is more, we investigated voluntary alcohol consumption in *Per2*^{Brdm1} mice with the results suggesting a relationship between this circadian clock gene and ethanol consumption.
Objective To further complement the *mPer2* study, our lab proceeded to assess *mPer1*'s possible role on alcohol intake using operant and free choice two bottle paradigms.
Methods Using operant conditions, *Per1*^{Brdm1} and wild type mice were trained to self-administer ethanol (10%) under a fixed ratio 1 (FR1) paradigm. This was ensued by a progressive ratio (PR) schedule. Furthermore, extinction sessions were introduced, followed by reinstatement measures of ethanol-seeking behavior. In another set of animals, the mice were exposed to voluntary long-term alcohol consumption, ensued by a 2-month deprivation phase, after which the alcohol deprivation effect (ADE) was measured.
Results Mutant mice did not display a significantly divergent number of reinforced lever presses (FR1 and PR) than wild type animals. Furthermore, no significant differences between groups were obtained regarding reinstatement of ethanol-seeking behavior. Similar results were obtained in the two bottle free choice paradigm. Specifically, no genotype differences concerning consumption and preference were observed over a broad range of different ethanol concentrations. Moreover, after the deprivation phase, both groups exhibited significant ADEs, yet no genotype differences.
Conclusions Contrary to the *mPer2* data, the present findings do not suggest a relationship between the circadian clock gene *mPer1* and ethanol reinforcement, seeking, and relapse behavior.

Keywords Clock genes · *Per1* · Ethanol reinforcement · Ethanol reinstatement · Alcohol deprivation effect · Craving · Relapse

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Introduction

It is well known that many physiological functions of an organism undergo cyclical changes, subject to a circadian rhythm. Some of these physiological parameters include core body temperature, circulating hormones, feeding, and drinking behavior (Gachon et al. 2004; Rutter et al. 2002). Furthermore, and in this line of context, alcohol consumption displays diurnal rhythmicity as well, with animals voluntarily consuming more alcohol during the dark, rather than light phase (Danel and Touitou 2004). The endogenous

circadian program exhibits a periodicity of approximately 24 h, controlled by a biological clock, located in the suprachiasmatic nucleus (SCN), known to govern behavioral, physiological, and hormonal processes in synchronization with internal as well as external cues. The molecular components of this circadian clock include genes such as *Cry1*, *Cry2*, *Per1*, and *Per2* (Reppert and Weaver 2002). *Period* was the first circadian clock gene to be identified in *Drosophila* (Bargiello et al. 1984; Reddy et al. 1984). Not long after, *Period* homologues in the form of *mPer1*, *mPer2*, and *mPer3* were discovered in mice, of which the former two's protein products are thought to be involved in an autoregulatory loop driving the oscillatory machinery (for review, see Albrecht 2002; Albrecht and Eichele 2003). In addition to the master clock of the SCN, this molecular mechanism is believed to exist in the local or "slave" clocks of a majority of brain regions and peripheral tissues, which are in turn synchronized by the master clock itself (Gachon et al. 2004).

Previously, our lab demonstrated the stark difference in the opposing manner of involvement of *mPer1* and *mPer2* in the modulation of cocaine sensitization and reward. Thus, after a course of repeated cocaine administrations, $Per2^{Brdm1}$ mice exhibited a hypersensitized response in comparison to wild type littermates, whereas $Per1^{Brdm1}$ mice failed to display any sensitization at all to such a treatment (Abarca et al. 2002). What is more, we then proceeded in investigating alcohol-drinking behavior in $Per2^{Brdm1}$ mice, identifying alterations in the glutamatergic system of these animals, with the consequence of enhanced alcohol consumption (Spanagel et al. 2005). To further complement the latter finding, we proceeded to investigate the role of *mPer1* on alcohol intake using operant and free choice two bottle paradigms. Collectively, our results of the present study do not suggest a relationship between the circadian clock gene *mPer1* and ethanol-drinking behavior.

Materials and methods

Animals The wild type and $Per1^{Brdm1}$ mutant animals used in this study were littermates derived from intercrosses between heterozygous $Per1^{Brdm1}$ mice on a $129SvEv^{Brd}$/C57/BL6-$Tyr^{c\text{-}Brd}$ background. Eight- to ten-week-old male *mPer1* mutant and wild type mice were used in our experiments (Zheng et al. 2001). The animals were singly housed with food and water ad libitum. Artificial light was provided daily from 6 a.m. [Zeitgeber time (ZT)] to 6 p.m. (=ZT12) (12 h of light/12 h of darkness = LD 12:12 cycle) with room temperature and humidity kept constant (temperature 22+ 1°C; humidity 55+5%). The experiments were approved by the Committee on Animal Care and Use of the relevant local governmental body and carried out in accordance with the German Law on the Protection of Animals.

Apparatus Operant experiments were carried out using eight skinner boxes (TSE Systems, Bad Homburg, Germany), running on OBS software (TSE, Bad Homburg, Germany). Each box contained two ultrasensitive levers (required force <1 g), one designed as "active" and the other as "inactive". When the programmed ratio requirements were met on the active lever, 10 µl of the ethanol solution were delivered into a microreservoir. The delivery was accomplished within a second and a cue light located above the microreservoir was turned on during the delivery time.

Training and testing were performed during the active phase at ZT16, with individual sessions lasting 30 min. The reasons for choosing ZT16 (4 h into the dark cycle) are for one, mice (nocturnal animals) are well known to significantly drink more alcohol in the active (dark) phase (Hiller-Sturmhofel and Kulkosky 2001) and secondly, for consistency purposes, as all our operant experiments are conducted at this time such as the operant experiments in the Per2 study (Spanagel et al. 2005), thus permitting us to confidently compare and contrast this batch of animals with others.

Blood alcohol concentration Six wild type and six littermate $Per1^{Brdm1}$ mutant mice were injected with ethanol (20% *v*/*v*) at a concentration of 3.5 g/kg (i.p.). Tail vein blood samples (25–30 µl) were then collected at several time points (30, 60, 120, and 240 min) after the time of the injection. Finally, blood alcohol concentrations were determined using the nicotinamide dinucleotide phosphate enzyme spectrophotometric method (Rolf Greiner BioChemica GmbH, Germany).

Operant experiments

Alcohol-drinking behavior Animals (seven wild type, and seven littermate $Per1^{Brdm1}$ mutants) were trained to orally self-administer ethanol (10%) after having undergone a standard sucrose fading procedure using a fixed ratio 1 (FR1) paradigm. The time line of operant self-administration consisted of an initial 10 days of sucrose (5%) solution, followed by 5 days of sucrose (5%) and ethanol (5%), 5 days of ethanol (5%), 5 days of sucrose (5%) and ethanol (8%), 5 days of ethanol (8%), 10 days of sucrose (5%) and ethanol (10%) and, finally, ethanol (10%) only was introduced. The 10% ethanol session using the FR1 paradigm lasted for a total of 10 days.

Break point After the FR1 session, as described above, which ended with providing a stable baseline, the same animals were tested for a single session on a progressive-ratio

(PR) schedule for ethanol (10%) reinforcement, in which the response requirements increased by a step size of 2. That is, to receive a reward (light and the 10% ethanol solution), the animals had to press the lever two times more than the preceding event (1, 3, 5, 7, etc.). The final ratio completed in the 30-min session was defined as the break point.

Reinstatement After PR measurements, the same animals were further trained (for another 4 weeks) until a stable baseline was achieved. More specifically, the mice completed an additional 20 ethanol self-administration sessions under an FR1 reinforcement schedule. Once the baseline was attained, and to achieve extinction of this behavior, the active lever was deactivated. Therefore, after responding, no light went on, as well as no liquid being dispensed. Upon attainment of extinction, the active lever was again coupled with the light cue so as to examine potential cue-induced reinstatement.

Free-choice two-bottle drinking behavior and the alcohol deprivation effect Ten *Per1*^{Brdm1} mutant mice and nine littermate wild type mice were used in these experiments. After 1 week of habituation to the animal room, the animals were given continuous free access to increasing concentrations of aqueous ethanol solution. For the first 3 days, the animals were allowed to drink from two bottles containing tap water. After this, one of the bottles was changed to hold a 2% ethanol solution for 3 days. For the next 3 days, the alcohol bottle contained a 4% ethanol solution. This was followed by 9 days of an 8% and another 9 days of a 12% ethanol solution. Finally, the concentration was increased to 16% (*v*/*v*) for the subsequent 18 days. Then, the ethanol bottle was removed for a period of 2 months (deprivation phase), leaving the mice with the bottle containing tap water only. At the end of the deprivation phase, the 16% ethanol bottle was reintroduced alongside the water bottle and the alcohol deprivation effect (ADE) measured. The bottles, animals, and the food were weighed every day, with the position of the bottles swapped daily so as to avoid any preference to any one particular side. The alcohol intake in grams was obtained from the difference in the weight of the bottles from one day to the next, taking into account alcohol density (0.8 g/ml). Finally, the amount of alcohol ingested was expressed as the absolute amount of ethanol consumed with respect to the weight of the animal (grams of ethanol per kilogram).

Data analysis

All data are presented as means + the standard error of the mean (SEM) and a significance level of $p<0.05$ was used throughout this study. Mean comparisons were performed by using a Student's *t* test for dependent or independent samples or a two-way ANOVA for repeated measures, when necessary.

Results

Operant ethanol self-administration To study the reinforcing properties of alcohol in *Per1*^{Brdm1} mice, we carried out operant self-administration with an FR1 using a two-lever paradigm. After a sucrose fading procedure, the animals achieved stable lever responding for 10% ethanol. At this stage (approximately 20 weeks old), the wild type (wt) and *Per1*^{Brdm1} mutant mice weighed 28.6 g±0.7 and 27.7 g± 0.9, respectively, and thus displaying no significant difference in body weight. Both wild type and *Per1*^{Brdm1} mutant mice displayed an average of 30 reinforced lever presses per session (30±1.3 and 29.4±1.6 for wt and *Per1*^{Brdm1}, respectively, which translates into ethanol consumed in grams per kilogram as 1±0.05 and 1±0.08, respectively. Our previous experiments and data base show that an intake of about 1 g/kg of ethanol in a 30-min session corresponds to blood alcohol levels in the form of 25–40 mg/dl, which is in a range that produces pharmacologically relevant effects. This is in agreement with data from the literature using the same paradigm [e.g., Roberts et al. (2001); Fig. 1]. However, regarding the nonreinforced lever, a much lower number of responses was observed (12.4+0.7

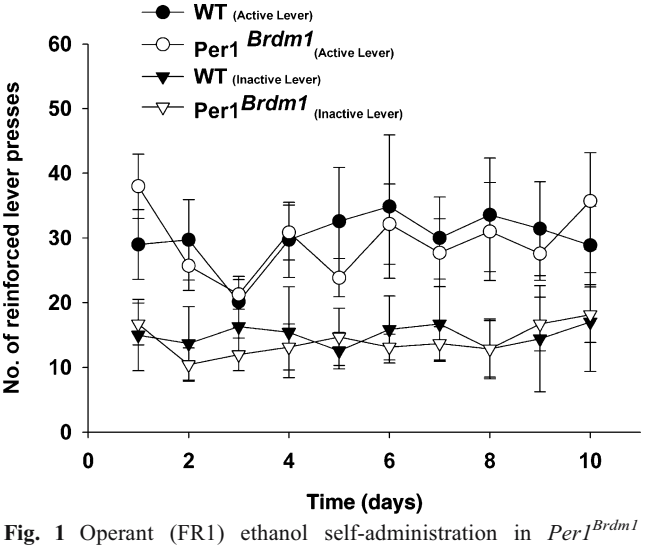

Fig. 1 Operant (FR1) ethanol self-administration in *Per1*^{Brdm1} mutants and wild type (*wt*) littermate mice. Data depict mean + SEM of reinforced (*circles*) and nonreinforced (*triangles*) lever and presses for each group. Two separate Student's *t* tests comparing the average of reinforced and nonreinforced lever presses, revealed that both genotypes displayed a significant number of operant responses on the ethanol-reinforced lever [$t(6)=4.6$, $p<0.01$ and $t(6)=3.4$, $p<0.02$ for wt and *Per1*^{Brdm1}, respectively]. However, the two genotypes did not show a significant difference in the number of responses on the active ($p=0.8$) or inactive ($p=0.3$) lever

and 16.0+0.9, for wt and *Per1*^{Brdm1} mutant mice, respectively). Two separate Student's *t* tests for dependent samples comparing the average of reinforced and non-reinforced lever presses for each genotype, revealed that both groups displayed a significantly higher number of operant responses on the ethanol-reinforced lever [$t(6)=4.6$, $p<0.01$ and $t(6)=3.4$, $p<0.02$ for wt and *Per1*^{Brdm1}, respectively]. However, the two genotypes did not differ from each other regarding operant alcohol self-administration. Thus, on applying a two-way ANOVA for repeated measures analysis (genotype×days), the two groups failed to show a significant difference in the number of responses on the active lever ($p=0.8$) (Fig. 1). Similar results were obtained, when using another two-way ANOVA for repeated measures to analyze the number of responses on the inactive lever ($p=0.3$) (Fig. 1). In both cases, the factor "days" as well as its interaction with the genotype failed to reach statistical significance, indicating that operant ethanol self-administration was stable in both groups across sessions.

Break point To investigate, if perhaps the incentive motivation for alcohol drinking in *Per1*^{Brdm1} mutant animals might differ, we proceeded with the application of a progressive ratio PR2 (response requirements increased by a step size of 2). Our results show that the break point (final ratio completed) did not significantly differ between the two genotypes, with the *Per1*^{Brdm1} animals not displaying any unusual incentive motivation for drinking ethanol ($p=0.6$) (Fig. 2).

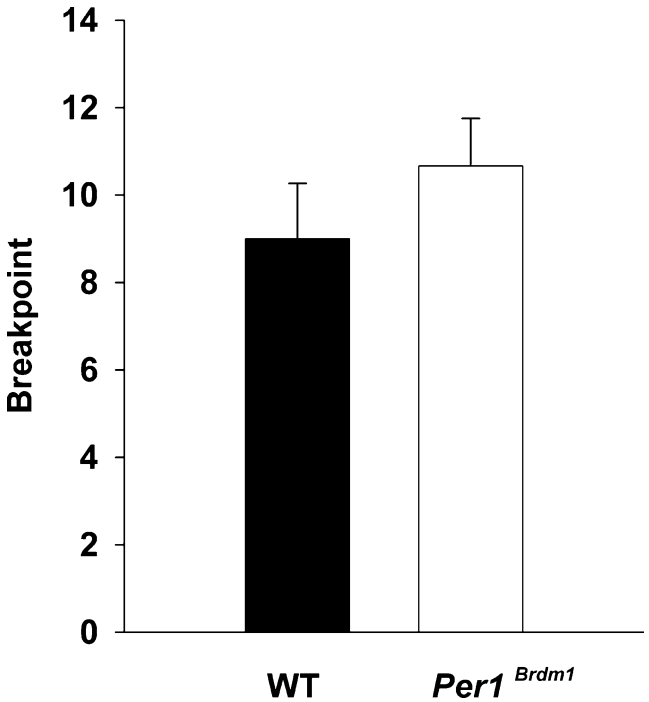

Fig. 2 Break point reached by *Per1*^{Brdm1} mutants and wild type (*wt*) littermate mice during operant ethanol self administration under a progressive ratio (PR) schedule (step size $n+2$). A single PR session was performed and the data analyzed using a *t* test for independent samples, revealing no significant effect of the genotype ($p=0.6$)

Extinction and cue-induced reinstatement of ethanol-seeking behavior To study reinstatement behavior in *Per1*^{Brdm1} mutant mice, we allowed the animals to acquire a stable FR1 lever responding for 10% ethanol. This was followed by an extinction period and a 1-day reinstatement procedure. Both groups of mice showed significant extinction over time. Thus, a two-way ANOVA (days × genotype) revealed only a significant effect for the "days" factor [$F(24,288)=8.2$; $p<0.001$]. However, the genotype factor or its interaction with the "days" failed to reach statistical significance, revealing that across sessions, both genotypes showed a similar reduction in the number of responses on the active lever (Fig. 3). Furthermore, wild type as well as *Per1*^{Brdm1} mutant mice displayed a significant day-effect for cue-induced reinstatement of ethanol-seeking behavior [$F(1,12)=42.2$; $p<0.001$], but again, a genotype effect was not present (Fig. 3).

Free choice two bottle alcohol-drinking behavior and ADE Differences in alcohol consumption and preference for the two genotypes are shown in Fig. 4. The mice were exposed to increasing concentrations of alcohol, and after 45 days they underwent a 2-month deprivation period, after which the animals were reexposed to ethanol (16% *v*/*v*) (Fig. 5).

Although *Per1*^{Brdm1} mice showed an increased dose-dependent alcohol-drinking behavior, the tendency was to drink less than their wild type littermates at low concentrations (2, 4, and 8%). At higher concentrations (12 and 16%), the graph shows slight increases in the amount of alcohol consumed by the *Per1*^{Brdm1} mutants relative to the wild type group. However, a two-way ANOVA revealed only a significant effect of the different concentrations of alcohol consumed [factor concentration: $F(4,68)=13.3$, $p<0.0001$ and $F(4,68)=40.3$, $p<0.001$, for intake and preference, respectively]. However, no significant differences were found between genotypes regardless of the concentration tested.

To further complement the reinstatement experiment, we proceeded to study the effects of a 2-month alcohol deprivation phase using a two-bottle paradigm. After the deprivation phase, both groups of animals displayed a classical ADE, consuming significantly more alcohol after the deprivation phase than before [$F(5,85)=19.6$; $p<0.001$]. However, there was no genotype effect ($p=0.8$), further demonstrating that *Per1*^{Brdm1} mutants do not differ from wild type mice in this respect.

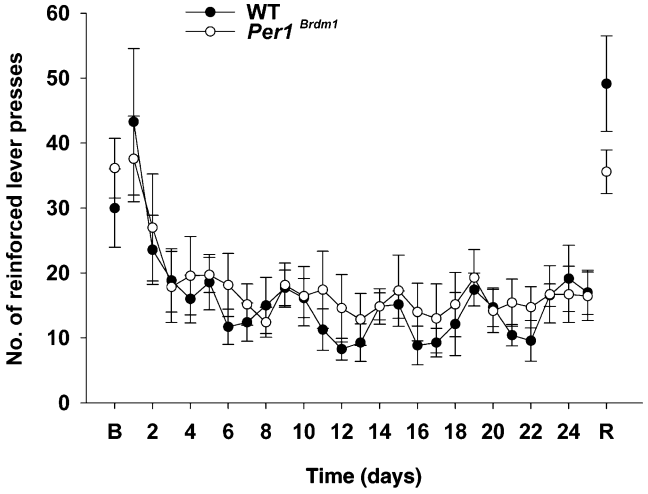

Fig. 3 Extinction and reinstatement of operant ethanol self-administration in *Per1*^{Brdm1} mutants and wild type (*wt*) littermate mice. Data depict mean + SEM of reinforced lever presses for each group. Further, *B* and *R* on the *x*-axis denote baseline and reinstatement, respectively. Regarding extinction, a 2-way ANOVA for repeated measures was performed (genotype × days), only yielding significance for the "days" effect [$F(24,288)=8.2$; $p<0.001$]. This reveals that the extinction procedure was equally effective for all groups. Furthermore, when the operant contingency was reestablished (reinstatement), both groups of mice reinstated ethanol-seeking behavior. Thus, when comparing the number of lever presses on the last day of extinction to that of the reinstatement day, using a 2-way ANOVA (day × group), only a significant "day" effect was observed [$F(1,12)=42.2$; $p<0.001$]

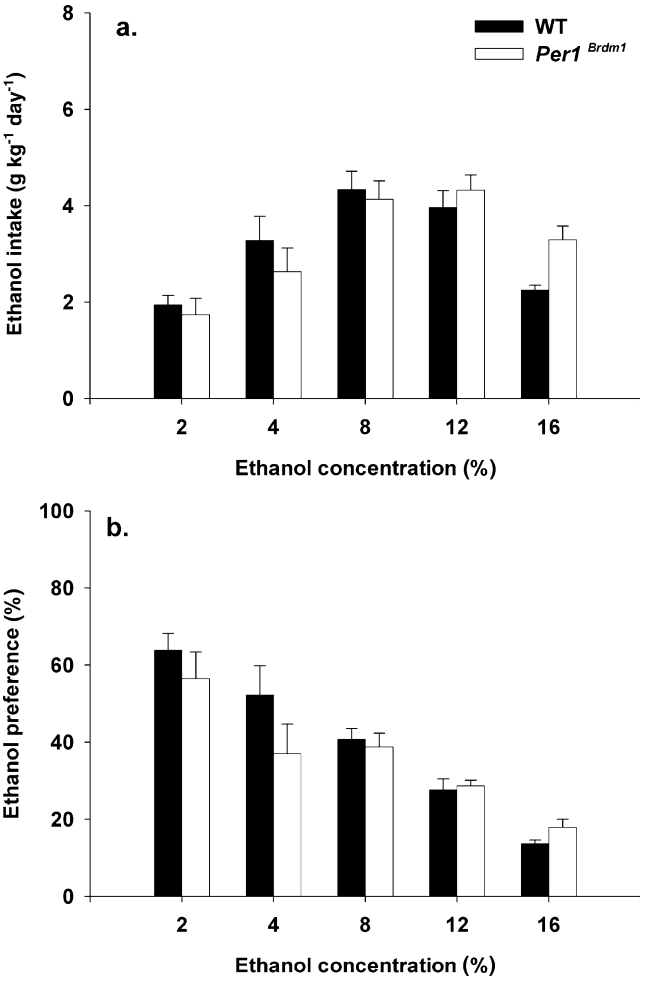

Fig. 4 **a** Intake and **b** preference of different alcohol solutions at increasing concentrations for *Per1*^{Brdm1} mutants and wild type (*wt*) littermate mice. A 2-way ANOVA revealed a significant interaction between the genotype and the different concentrations of alcohol consumed [factor concentration $F(4,68)=13.3$, $p<0.001$ and $F(4,68)=40.3$, $p<0.001$, for intake and preference, respectively]. However, no significant differences were found between these groups at all the doses used

Blood alcohol concentration An analysis of blood ethanol concentration was carried out to determine, whether there are any differences in the metabolism of alcohol in *Per1*^{Brdm1} mutants and their wild type littermates. After an injection of ethanol, blood samples were collected at several time points later and the concentration of ethanol contained within analyzed (Fig. 6). A temporal analysis using a two way ANOVA for repeated measures revealed no significant difference between the two groups of animals ($p>0.05$).

Discussion

In the present study, we suggest that *Per1*^{Brdm1} mutant animals and wild type littermates do not differ with regard to alcohol-drinking behavior. Furthermore, there is no genotype difference for alcohol-seeking and relapse behavior. This comes in contrast to a recent study by our lab, in which we established that *Per2*^{Brdm1} mutant mice consume more ethanol than their wild type littermates and that genetic variations of the human *Per2* gene are associated with increased alcohol intake. The enhanced alcohol consumption in *Per2*^{Brdm1} mutant mice is due to a hyperglutamatergic state as a result of impaired glutamate transport (Spanagel et al. 2005). This, however, seems not to be the case with a dysfunctional *Per1* gene. Thus, deletion of the *mPer1* gene does not result in alterations of glutamatergic components as revealed by differential display analysis of the brains of *mPer1* and wild type mice (unpublished observations). As well, it does not lead to any differences in the metabolism of alcohol. Furthermore, it does not lead to a "behavioral alcohol phenotype". In fact, a wide array of behavioral experiments was conducted to examine alcohol-drinking behavior in *Per1*^{Brdm1} mutant mice without, in the end, identifying any aberrations in alcohol consumption. Moreover, body mass variations were absent, showing again that *Per1*^{Brdm1} mutant mice do not differ from their wild type counterparts. This observation disagrees with the finding by Dallmann et al. (2006), where

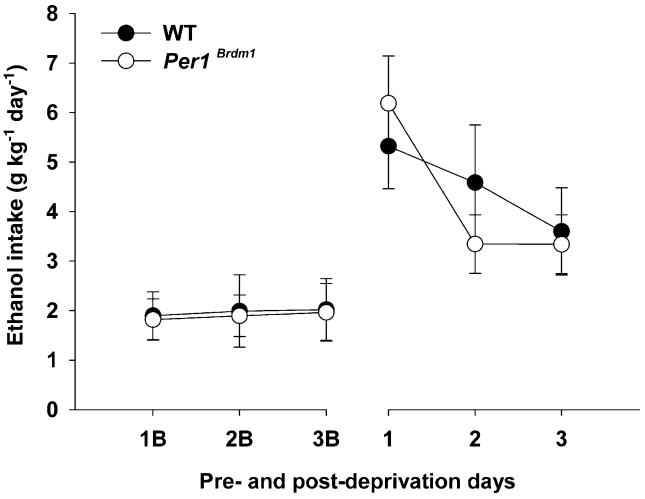

Fig. 5 Alcohol-drinking behavior before (denoted by the *B days* on the *x*-axis) and after the 2-month deprivation phase in *Per1*^{Brdm1} mutants and wild type (*wt*) littermate mice. Statistical analysis using a 2-way ANOVA for repeated measures revealed only a significant effect for the different concentrations of alcohol consumed [factor concentration $F(4,68)=13.3$, $p<0.0001$ and $F(4,68)=40.3$, $p<0.001$, for intake and preference, respectively]. However, no significant differences were found between genotypes, regardless of the concentration tested

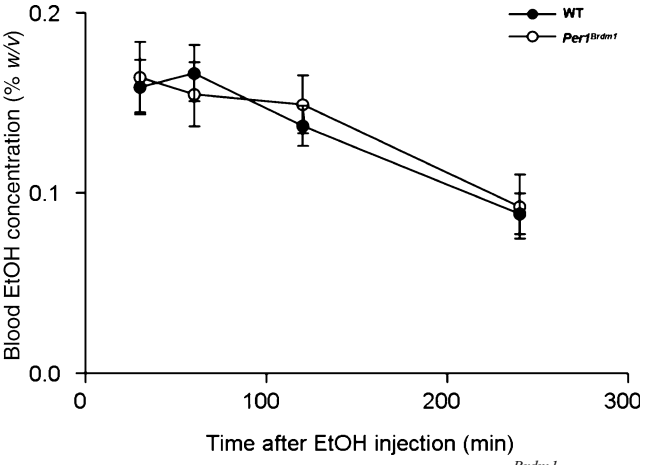

Fig. 6 Blood alcohol concentration curves from *Per1*^{Brdm1} mutants and wild type (*wt*) littermate mice. After having been injected with ethanol (20% *v/v*) at a concentration of 3.5 g/kg (i.p.), blood samples were collected and analyzed at several time points. A 2-way ANOVA for repeated measures was performed (genotype × time point), revealing no significant effect of the genotype ($p>0.05$)

mice lacking *Per1* displayed lighter body mass and attributing this and other differences to be possibly due to an impaired corticosterone rhythm in *mPer1* animals. In our experiments, we set out using operant chambers to examine the reinforcing properties of ethanol self-administration. In this paradigm, the attainment of a drop of ethanol is contingent upon the completion of pressing the lever once (FR1). This FR1 ratio is similar in its consequences to the home cage-drinking paradigm. In both cases, wild type and *mPer1*^{Brdm1} animals did not significantly differ from each other with regard to alcohol consumption. Furthermore, the different concentrations of alcohol presented to the animals in their home cages did not lead to any divergent drinking patterns, except that at the highest concentration (16%) enhanced alcohol intake in *mPer1*^{Brdm1} animals was observed. This could reflect a difference in the aversive taste properties of high concentrated alcohol solutions. Nevertheless, this effect faded away during an 18-day measurement period (see baseline consumption for ADE measurements). With these results in mind, we took the study further, curious to see the effect of an increase in response requirement, that is, changing the FR1 to a progressive ratio PR2, where the response requirement was increased by a step size of 2. Again, the animals did not display dissimilar behavior in their motivation for seeking alcohol with both groups exhibiting a similar break point and thus showing no difference in the final ratio completed.

Having observed no aberrational performance regarding the motivational facet, we decided to investigate ethanol-seeking (craving) and relapse, behavioral traits, which aid in measuring aspects of human alcohol dependence, using the reinstatement and alcohol deprivation models (Stewart and de Wit 1987; Sanchis-Segura and Spanagel 2006). After having completed a stable baseline in alcohol consumption, both groups underwent successful extinction. It is important to note that extinction in mice can be problematic, in the sense that a substantial number of lever responses may still be observed even after extensive extinction. The reason for this phenomenon is that lever pressing in mice is reinforcing per se (Spanagel and Sanchis-Segura 2003). After a substantive extinction period, all animals were reexposed to the ethanol-associated cue on the day of reinstatement in the operant chambers. Even though, the two groups did not show any genotype associated significant differences before and during reinstatement, the wild type and mutant mice did successfully reinstate ethanol-seeking behavior at considerably high rates. Up until this study, reinstatement models had only been performed with rats. To our knowledge, this is the first study displaying successful reinstatement of alcohol-drinking behavior in mice.

Importantly, the behavior of human alcoholics is characterized by high rates of relapse to alcohol use, even after substantially long periods of abstinence. To more fully understand this behavior, experiments using animal models employing the ADE have been performed, demonstrating that appropriately conducted deprivation phases lead the animals to a pronounced but temporary rise in alcohol intake, which is interpreted as a relapse-like drinking

behavior (Sanchis-Segura and Spanagel 2006). Thus, to examine ADE in the two groups, the mice were exposed to a 2-month deprivation period. An analysis of the results shows that alcohol consumption after the deprivation period is significantly enhanced in both groups of animals, thus displaying a classical ADE. However, both groups did not differ appreciably from each other, thus ruling out an involvement of *mPer1* in ADE.

The existence of circadian rhythmicity with regard to drinking behavior has already been very much established in animals, especially in the form of enhanced drinking during the dark phase (Freund 1970; Possidente and Birnbaum 1979; Poirel and Larouche 1986). Furthermore, it has been shown that lesions in the SCN lead to a loss of the circadian program with respect to drinking behavior, clearly demonstrating the vital role of the master clock in this regard (Stephan and Zucker 1972). Per1 and Per2 are two essential components of circadian clocks, involved in driving recurrent circadian rhythms. Previously, we established that the *Per2* gene is involved in aberrantly enhanced alcohol consumption (Spanagel et al. 2005). In this study, and collectively examining the results, it may be concluded that the circadian gene *mPer1*, and unlike *mPer2*, does not demonstrate a role in alcohol-drinking behavior in mice.

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